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Enrichment of High-Value Chemicals from Natural Product Matrices

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Introduction

The extraction and fractionation of high value chemicals from natural product matrices, using critical fluid technology, is seeing increasing use particularly with regard to consumer interest in nutraceutical products. To optimize processing conditions and final product specifications, it is traditional to run a series of experiments on bench scale equipment that requires considerable time and effort, before commencing with scale up of the separation process. This requires that a number of permutations and combinations of conditions be tried, although statistical optimization schemes have been applied to supercritical fluid extraction (SFE) [1,2].

Recently the field of combinatorial chemistry and science have employed a number of ingenious approaches for surveying reaction and production conditions for producing a resultant end product [3,4]. Such an approach should be amenable for establishing processing conditions with critical fluids, since automated instrumentation has been developed for routine analytical use [5,6]. Toward that end several investigators have used available instrumentation to rapidly expedite process optimization. Montanari, et al. [7,8] have studied the extraction of phospholipids from soybeans and isolation of shea butter extracts using this rapid approach, while Frykman, et al. [9] have screened enzyme activities in the presence of supercritical carbon dioxide for potential as transesterification catalysts (SC-CO_2).

In this study we have extended the above concept for developing a fractionation scheme using SFE in combination with preparative supercritical fluid chromatography (SFC) for the isolation of phospholipid (PL) concentrates enriched in a specific component. As a part from optimizing the SFE separation stage, we have utilized an automated SFE instrument to establish the essential parameters required to conduct sequential elution chromatography utilizing SC-CO_2 as a component in the mobile phase. In addition, the extraction of cedarwood oil (CWO), a high value oil at low levels in its natural matrix, has been optimized with respect to extraction pressure, temperature, moisture in the wood matrix, and aging of the sample.

Materials and Methods

Phospholipid Fractionation. Phospholipid standards of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). A soybean-based lecithin sample was acquired from Central Soya (Fort Wayne, IN).

Supercritical fluid fractionation (SFF) and sorbent screening studies were performed with an Isco Model SFX 3560 automated extractor (Isco, Inc., Lincoln, NE) and utilizing the above-mentioned lecithin. The sorbents used in the SFF studies were as follows: silica gel (60-200 mesh) and Celite 545 (J.T. Baker Chemical Co., Phillipsburg, NJ), amino-bonded silica (Varian Associates Inc., Harbor City, CA), neutral and acidic alumina (60-325 mesh, Fisher Scientific, Fair Lawn, NJ), basic aluminum oxide (~150 mesh, Aldrich Chemical Co., Inc., Milwaukee, WI), Alumina C (Universal Scientific, Inc., Atlanta, GA), Chem Tube-Hydromatrix (Varian Associates Inc., Harbor City, CA) and bentonite (200-325 mesh, Oil Dri Corporation of America, Chicago, IL).

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Sorbents were added to a 10 mL extraction vessel and lecithin (0.5 g) was manually applied to the top of the sorbent bed. The extraction/fractionation testing procedure was then commenced with fractions being collected at 60 minute (120 mL solvent) intervals. The first 2-3 fractions effectively resulted in removal of any residual oil from the lecithin. The parameters for subsequent fractions were then designed to enrich and separate the individual PPLs. A typical elution sequence is shown in Table 1 below.

Fraction 1-2	65 MPa	80°C	60 min	2 mL/min	CO_2
Fraction 3	35 MPa	50°C	60 min	2 mL/min	10 vol% modifier/CO_2
Fraction 4-6	35 MPa	50°C	60 min	2 mL/min	20 vol% modifier/CO_2
Fraction 7-9	50 MPa	50°C	60 min	2 mL/min	20 vol% modifier/CO_2
Fraction 10-12	50 MPa	50°C	60 min	2 mL/min	25 vol% modifier/CO_2
Fraction 13-14	65 MPa	50°C	60 min	2 mL/min	30 vol% modifier/CO_2

Table 1: Conditions for the elution sequence of phospholipids from various sorbents.

All analyses for phospholipids were performed by high performance liquid chromatography (HPLC) using a Spectra-Physics SP8800 pump (Thermo Separation Products, Fremont, CA), a Rheodyne 7125 loop injector (200 µL), a Bio-Rad Model 1250424 column heater (Bio-Rad, Inc., Richmond CA) @ 35°C, an Alltech Model 500 ELSD evaporative light-scattering detector (ELSD) at 90°C and 2 SLPM of N_2 (Alltech Associates, Inc., Deerfield, IL), and an SP Datajet integrator (Thermo Separation Products, Fremont, CA). The HPLC column was a Lichrosphere Si 60/II, 3 µm particle size, 250 x 4 mm (EM Separations, Gibbstown, NJ). The mobile phase was a linear gradient of A:B (80:20) to A:B (20:80) over 30 minutes at a flow rate of 0.7 mL/min . The composition of solvent A was chloroform:tert-butyl methyl ether (750:150, v:v), and solvent B was methanol:ammonium hydroxide:chloroform (920:70:10, v:v:v).

Cedarwood Oil Extraction. Cedarwood chips used in this study were prepared from a kiln-dried cedar board purchased from a local lumber mill. Immediately after making the chips, they were packaged in a zipper-lock plastic bag, then wrapped in aluminum foil and stored at -70°C until used for experiments. A commercial sample of CWO was purchased from Aldrich (Milwaukee, WI), (-)-α-cedrene, (+)-β-cedrene, (-)-thujopsene, (+)-cuparene, and (+)-cedrol were purchased from Fluka Chemika (Switzerland).

The SFE experiments were also conducted with the ISCO Model SFX 3560 extractor. In these extractions, approximately 2.4 g of sample was added to the extraction cell and secured between glass fiber filter disks (8 mm dia, Leco Corporation, St. Joseph, MI) on both the top and bottom of the cell. Eighteen extraction conditions were evaluated consisting of all possible combinations of three extraction temperatures (40, 70, and 100°C) and six extraction pressures (10.3, 19.0, 27.6, 41.4, 55.2, and 69.0 MPa) (see Table 2). For all of these extractions, the extraction sequence was 1 min static hold followed by a 25 min dynamic extraction using a flow of 2 mL/min CO_2. The variable restrictor was heated to 80 °C and extracts were collected in 20-mL pre-cooled (O°C), pressurized vials. SFE/SFC-grade CO_2 (Air Products and Chemicals, Inc., Allentown, PA) was used in all of the above SFE experiments, including the phospholipid fractionations. Each CWO extraction at the various conditions was replicated twice.

Since the collected extracts also contained water in addition to the CWO, it was necessary to determine each component in the resultant extract. The determination of the amount of CWO was not possible by direct weighing of the collected extract, therefore the following procedure was

adopted. Approximately 0.5 g of anhydrous sodium sulfate, 1 mL of water saturated with sodium sulfate and 2 mL diethyl ether were added to the collection vial and mixed thoroughly. The ether layer containing the CWO was removed and transferred to a tared vial. The collection vial was re-extracted twice with 2 mL ether and the combined ether extracts were concentrated under a gentle stream of nitrogen until there was no detectable weight loss with further drying. The weight of the CWO extract was then determined and the percent CWO extracted calculated based on the original sample weight.

Chemical analysis of the CWO extracts by gas chromatography and GC/MS were conducted as follows. A solution of the CWO in hexane (ca. 200 ng/μL) was prepared and analyzed by gas chromatography (GC) to determine the percentage contribution of the individual components [10]. CWO extracts were analyzed by 0.5 min split-delay splitless injection onto a Hewlett-Packard 5890 Series II GC equipped with a flame ionization detector. The separations were performed on a SP-2380 column (60 m, 0.25-mm dia, 0.20 μm film thickness) (Supelco, Bellefonte, PA) using He as the carrier gas at a linear flow velocity of 18 cm/sec. The temperature program was 60°C for 1 min, 5°C/min to 250°C, with an isothermal hold for 1 min. The injector and detector temperatures were 235°C and 250°C, respectively. Injections were made using a Hewlett-Packard Model 7683 auto-injector and sample volumes were 1 μL. The chromatographic data were acquired using a Hewlett-Packard Vectra VL2 computer and ChemStation software. Retention indices were determined relative to *n*-alkane standards [11]. Electron impact mass spectra were obtained using a Hewlett-Packard 5971 mass selective detector using an ionization potential of 70 eV. Sample introduction was through a Hewlett-Packard 5890 GC with a SP-2340 column, using the above conditions as described for the GC analyses.

The effect of extraction time was examined by comparing twelve extraction times varying from 5 min to 60 min in 5 min increments. The extraction temperature was 100°C and the pressure was 27.6 MPa, with the extractor restrictor at 100°C. Extracts were collected as described previously and the cedarwood chips were weighed after each extraction to determine their weight loss with extraction. The cells were also allowed to cool to room temperature for ca. 1 hour as well as to allow the dissipation of any imbibed CO_2 from the chips. Each run for a given extraction time was replicated twice and the average reported in Table 3.

During the previous experiments, it was noted that the concentration of the hydrocarbon sesquiterpenes, cedrene and thujopsene, were quite low in comparison to the concentrations of the sesquiterpene alcohol, cedrol, and that they were much lower than previously reported values [12, 13]. It was hypothesized that during the time between production of the chips and extraction that some of the CWO had been lost from the wood matrix. Therefore, experiments were conducted to determine if the time between chipping and extraction had an effect on the yield of CWO, as well as the oil composition. Approximately 20 g of freshly prepared cedarwood chips were separated from a large batch and approximately 5 g were immediately separated and sealed in a zipper-lock plastic bag, enclosed in aluminum foil and stored at -70°C. The remaining chips were placed in a 190 mm dia X 100 mm deep Pyrex® dish and placed on a bench in the laboratory at ambient temperature to age. Five gram samples were removed from the Pyrex® dish, packaged and stored at -70°C after 1, 2, and 3 weeks on the lab bench. Then 2 g sub-samples from each of the four treatments (i.e., 0, 1, 2 and 3 week-old chips) were extracted as described above at 100°C, 27.6 MPa, for 25 minute dynamic extraction periods and analyzed using the conditions described above. For this purpose, two replications of each batch of aged chips were extracted and analyzed.

Temp. (°C)	Press. (MPa)	CO_2 Density (g/mL)	Mean Weight % Collected		Oil Composition (%)	
			Total	CWO	thujopsene	cedrol
40°C	10.3	0.66	5.1%	3.0%	16.2%	42.3%
40°C	19.0	0.84	6.3%	3.9%	16.6%	42.2%
40°C	27.6	0.91	6.4%	3.7%	16.0%	41.2%
40°C	41.4	0.97	6.3%	3.6%	16.1%	41.3%
40°C	55.2	1.02	6.4%	3.6%	15.6%	40.0%
40°C	69.0	1.05	6.2%	3.5%	16.2%	41.4%
70°C	10.3	0.27	4.5%	1.0%	19.7%	36.6%
70°C	19.0	0.63	8.4%	4.1%	15.1%	41.4%
70°C	27.6	0.77	8.3%	4.3%	16.1%	40.6%
70°C	41.4	0.87	8.9%	4.3%	16.1%	40.0%
70°C	55.2	0.93	8.8%	4.3%	16.0%	39.9%
70°C	69.0	0.97	7.5%	4.0%	15.9%	40.5%
100°C	10.3	0.20	6.9%	1.0%	20.4%	35.3%
100°C	19.0	0.44	10.3%	4.2%	14.8%	41.8%
100°C	27.6	0.63	10.3%	4.4%	16.0%	39.6%
100°C	41.4	0.77	10.4%	4.6%	16.0%	40.3%
100°C	55.2	0.85	10.2%	4.4%	15.5%	39.8%
100°C	69.0	0.89	9.8%	4.3%	15.7%	40.7%

Table 2: Effect of temperature and pressure on yield and oil composition of CWO.

Extraction Time (min)	Cedarwood Oil Composition (%)		
	α-cedrene	thujopsene	cedrol
5	1.4%	1.0%	67.1%
10	1.6%	1.1%	70.1%
15	1.7%	1.2%	69.5%
20	1.7%	1.2%	70.0%
25	1.8%	1.4%	70.0%
30	1.8%	1.4%	70.1%
35	1.9%	1.4%	70.0%
40	1.9%	1.5%	69.1%
45	1.9%	1.4%	69.7%
50	1.9%	1.5%	69.6%
55	1.9%	1.4%	69.3%
60	1.8%	1.4%	70.9%

Table 3: Effect of extraction time on CWO composition.

Results and Discussion

Phospholipid Fractionation. The purpose of the sorbent screening program was to allow a more accurate assessment of the ability to separate/fractionate PL-containing extracts rapidly on small-scale columns, which emulated the sorbent(s) that would be used in preparative chromatographic columns. Initial SFF experiments were performed with silica gel as the sorbent. An extraction cell was filled with sorbent and a soya-based lecithin was applied as described to the top (inlet) of the cell. The discrete fractions that were eluted using a sequential program as described in Table 1 were the analyzed by HPLC giving the ELSD profiles shown in Figure 1a. Figure 1a shows the profile of the starting lecithin, while Figure 1b (representing the 4th collected fraction), shows a decreased amount of PE compared to the original matrix. The results from the seventh fraction that was collected are shown in Figure 1c and indicate that it mainly consists of PC; with PI as only a minor constituent of the fraction, and the complete absence of PE and PA . This nicely illustrates the SFF effect being demonstrated on this particular sorbent.

SFF (with silica gel) was also attempted with 100% ethanol (EtOH) modifier. In this case, the PLs were strongly retained and elution did not occur within a reasonable time or volume of eluent fluid. Therefore water was added as a secondary modifier to the EtOH in order to affect PL elution. In this regard, SFF was attempted with an increasing percentage of water in the modifier (EtOH:H_2O; 7:3, v/v), however, this mobile phase composition did not produce any fractionation of PLs; it only affected a more rapid elution of the PLs. It was eventually found that all four PLs eluted in a single fraction when the extraction fluid concentration was 20 vol% modifier/CO_2.

By contrast to adjusting the modifier in the mobile phase with water, we attempted to deactivate the silica gel by addition of water to the sorbent. This was done at two different levels (5 and 20 weight %). With addition of water to the silica gel, it was hoped that 100% EtOH could be used as the modifier. However, addition of water to the stationary phase (silica gel) did not work as well as having water in the mobile phase. Interestingly, the silica gel, deactivated with 5 weight % water retained the PLs,, until a 40 vol% ethanol-modified CO_2 fluid was passed over the sorbent. Further, a concentration of 30 vol% EtOH/CO_2 was needed to elute PLs from the silica gel deactivated at 20 weight % with water. This may be compared to the results obtained when adding 20-25 vol% of modifier to CO_2, with the modifier being EtOH:water (9:1), using neat silica gel.

Sorbents other than silica gel were also investigated. All of the SFF conditions and fractionation sequences were kept identical to those reported above so that just the influence of the sorbent type could be examined combinatorially. Chem-Tube Hydromatrix and Celite 545 did not fractionate the PLs at all, but PL elution occurred at lower modifier concentrations than those required using silica gel; characteristic of a less adsorptive and selective sorbent . Amino-bonded silica was observed to fractionate the PLs, but required almost 40 vol% modifier/CO_2 to elute PC. Bentonite clay, a sorbent capable of chemisorbing PLs, exhibited such strong retention properties that PC did not elute even with 40 vol% modifier.

Four different types of alumina were also examined in the sorbent screening experiments. Alumina C was found to be inadequate for PC enrichment. Two of the collected fractions were enriched with PC, but early collected fractions also contained both PE and PC. Collection of later fractions indicated a carryover of PC into the fractions containing PI. Basic alumina proved capable of fractionating the PL moieties as well as silica gel. PE and PI were separated into early and late eluting fractions, respectively, but PC again eluted over a broad range of modifier concentration (10-30 vol%) and was found in nearly every fraction. Acidic and neutral aluminas behaved similarly and were slightly superior than silica gel in terms of fractionating the PLs. There was some minor co-elution of PC with PE, but PC eluted as a discrete profile with no cross-contamination from PI or PA.

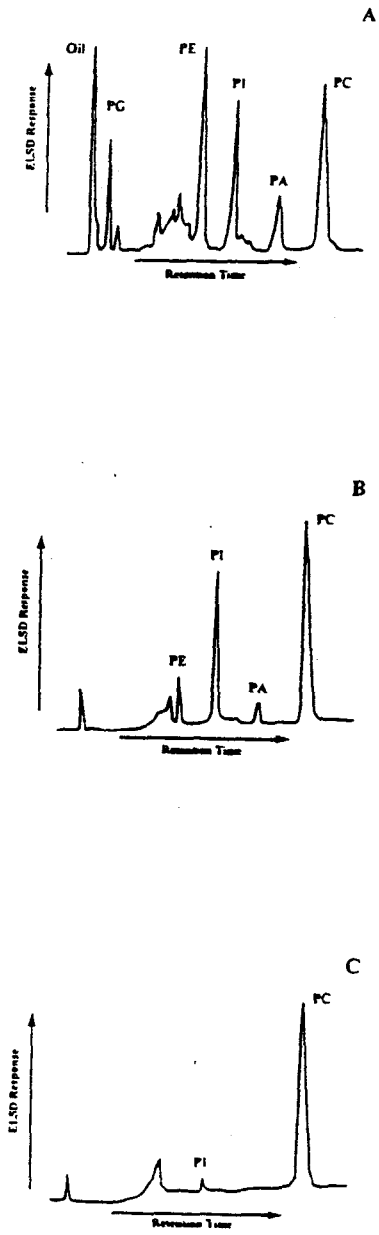

Figure 1. HPLC-ELSD chromatographic profiles of: (A) lecithin, (B) 4th fraction, and (C) the 7th fraction eluted from a silica gel test sorbent.

In conclusion, the above sorbent screening experiments permitted conditions to be established for SFF of lecithin, and ultimately, extracts derived directly from the SFE of soybeans. Using neutral alumina as the preferred sorbent, enrichment factors of 3.6 to 8.1 relative to their concentration in soya were recorded for the individual PLs. By collecting discrete fractions from the SFC process, PC, PE, and PI culd be obtained at purity levels of 56, 75, and 77 %, respectively.

Cedarwood Oil Extraction. Inspection of Table 2 reveals that the total amount of extract increases with increasing temperature and to a lesser degree with increasing pressure. Only at 10.3 MPa and 70°C or 100°C, did the amount of CWO also increase with temperature. This result is consistent with the low density of CO_2 which is not sufficient to permit extraction any appreciable amount of CWO. However it appears that a CO_2 density of at least 0.4 g/mL is required to extract most of the oil content of the wood matrix and that higher fluid density only results in a marginal increase in oil yield. Reverchon and Taddeo [14] noted that because sesquiterpenes and oxygenated sesquiterpenes are readily soluble in SC-CO_2, high fluid densities are not required for their extraction. Our results support this conclusion, in that some of the highest yields were not at the highest densities. For example, the combination of 100°C and 2,750 psi (density of 0.44g/mL), gave a relatively high CWO yield of 4.2 wt.%.

The amount of CWO reported to be present in *J. virginiana* cedarwood varies widely from 0.97- 3.5%. From Table 2, the highest yield observed was 4.6%, which is higher than previous reports, suggesting that SC-CO_2 is a very effective method for the extraction of CWO from cedarwood chips. However, the component ratios for the SC-CO_2 extracts (Table 1) were different from that reported in previous studies (15,16). The percent contribution of thujopsene to the CWO was ca. 16% for the SC-CO_2-derived oil in our study, while Adams (16) reported that thujopsene accounted for slightly over 27% of the oil. This difference may be a due to the fact that the cedarwood chips extracted in this study came from a kiln-dried board from which some of the oil had been lost, especially the more volatile hydrocarbons, such as thujopsene.

The yield of total extractives initially increased with extraction time, leveling of at ca. 25 minutes at just over 11 wt. %. Analysis of these timed extracts for the amount of CWO showed that it did not vary significantly with extraction time; essentially all of the CWO was extracted within the first 5 minutes from the sample. Therefore most of the mass extracted up to 25 minutes was apparently water. The length of extraction time had no apparent effect on CWO composition as shown in Table 3 for an aged cedarwood oil sample. For the extraction of fresher cedarwood oil samples, the percent contribution of thujopsene varied only from 15.2% to 16.2% and cedrol from 40.9% to 42.2% during the course of the extraction.

The effect of cedarwood chip age on total CWO yield showed that the weight of the total collected material actually increased with time between chipping and extraction. This is probably a result of the cedarwood chips adsorbing water from the air and this water subsequently being co-extracted with the CWO. However, on a dry CWO-basis, the mass of CWO actually decreases with increasing cedarwood chip age, a reflection of the loss of the oil from the wood matrix due to its volatility over time. It was also found that the relative percent of thujopsene decreased with chip age while that of cedrol increased with chip age. This indicates that the more volatile hydrocarbons, such as thujopsene and cedrene, are lost more quickly from the chips than the alcohol, cedrol. This suggests that the wood chips should be extracted as soon as possible after chipping to prevent the loss of these volatile compounds.

Conclusions

With the advent of computer controlled, automated SFE instrumentation, it has become possible to rapidly examine in a combinatorial fashion extraction, fractionation, and reaction

conditions for optimizing the associated processing conditions. In the reported studies, two types of schemes were studied and optimized: the selection of sorbents for SFF of PLs and the SFE of cedarwood oil. The use of this combinatorial approach can save considerable time and cost, and allow the study of the many variables associated with critical fluid extraction from natural matrices. Both sequential and parallel modes of combinatorial screening exist and should be exploited in the future in the various applications of critical fluid technology.

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